DOI:
10.1002/jnr.23911

Document Version
Peer reviewed version

Contribution of mast cells to injury mechanisms in a mouse model of pediatric traumatic brain injury

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Abbreviated Title: Mast cells in pediatric TBI

Associate editor: Eric Prager

Key words: Neuroinflammation, histamine, apoptosis, myelin, neuron, microglia

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Financial support information

This study was supported by grants from Inserm, Université Paris Diderot, Université Sorbonne-Paris-Cité, Investissement d'Avenir (ANR-11-INBS-0011, NeurATRIS), ERA-NET Neuron (Micromet), DHU PROTECT, PremUP, Fondation de France, Fondation pour la Recherche sur le Cerveau, Fondation des Gueules Cassées, Roger de Spoelberch Foundation, Grace de Monaco Foundation, Leducq Foundation, Cerebral Palsy Alliance Research Foundation Australia, and the Wellcome Trust (WSCR P32674) In addition, the authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. The supporting bodies played no role in any aspect of study design, analysis, interpretation or decision to publish this data.

Abstract

The cognitive and behavioral deficits caused by traumatic brain injury (TBI) to the immature brain are more severe and persistent than injuries to the adult brain. Understanding this developmental sensitivity is critical as children under four years age of sustain TBI more frequently than any other age group. One of the first events following TBI is the infiltration and degranulation of mast cells in the brain, releasing a range of immunomodulatory substances, and inhibition of these cells is neuroprotective in other types of neonatal brain injury. We investigated for the first time the role of mast cells in mediating injury in a P7 mouse model of pediatric contusion-induced TBI. We show that various neural cell types express histamine receptors, and histamine exacerbates excitotoxic cell death in primary cultured neurons. Cromoglycate, an inhibitor of mast cell degranulation, altered the inflammatory phenotype of microglia activated by TBI, reversing several change, but accentuating others, when administered before TBI. However, regardless of the time of cromoglycate administration, inhibiting mast cell degranulation did not affect cell loss, as evaluated by ventricular dilatation or cleaved caspase-3 labeling, or the density of activated microglia, neurons or myelin. In double-heterozygous cKit mutant mice lacking mast cells, this overall lack of effect was confirmed. Our results suggest that the role of mast cells in this model of pediatric TBI is restricted to subtle effects but that they are unlikely to be viable neurotherapeutic targets.

Significance statement

Traumatic brain injury is a leading cause of acquired disability and death in infants and children. We assessed mast cell (MC) stabilization as a therapeutic strategy in a model of pediatric TBI using the immature mouse. MCs infiltrated the brain post-TBI, neurons and glia expressed receptors for a histamine (an important factor released from MCs), and histamine aggravated neuronal excitotoxic death *in vitro*. However, manipulations of MCs *in vivo* with pharmacologic or genetically approaches revealed that MCs have marginal effects on TBI neuropathology. This suggests that the actions of MCs may be of less importance in the immature brain compared to the mature brain.

Introduction

Children under the age of four years sustain traumatic brain injury (TBI) more frequently than any other age group (Koepsell et al. 2011), and in children under two, rates of TBI serious enough to be fatal or to require intensive care support are as high as 50 per 100,000 (Keenan et al. 2003). This is of particular concern as the cognitive and behavioral deficits caused by TBI to the immature brain are more severe and persistent than those observed following comparable injuries to the mature (adult) brain (Anderson et al. 2005; Rivara et al. 2012), despite the superior potential for repair of the former (Bennet et al. 2013). This is likely due to the combined effect of disrupted normal development and the direct effects of injury. TBI in the pediatric population is caused by a diverse range of injuries and insults. These include acceleration/deceleration injuries (shaken baby syndrome) as well as contusion injuries (direct impact to the skull such as car accidents or falls), both inflicted and accidental (Pinto et al. 2012) .

The primary injury process in TBI is mechanical damage (i.e. shear forces inducing vascular damage and bleeding), followed by secondary pathological processes including excitotoxicity, ischemia and neuroinflammation (Hagberg et al. 2012; Xiong et al. 2013), which lead to mitochondrial dysfunction and cell death. Among the first events of the injury response, in both the adult and the developing brain, is the degranulation of mast cells (Stokely and Orr 2008), whereby these long-lived and self-replicating effectors of the immune system (Dropp 1979; Galli et al. 2005) release numerous inflammatory mediators such as histamine, cytokines, lipid metabolites and nitric oxide (Silver et al. 1996). Mast cells enter the brain in the early postnatal period as part of normal development (Panula et al. 2014). Mast cell degranulation is toxic to neurons and glia *in vitro* and *in vivo* (Dean et al. 2010;

Hendrix et al. 2013), while their stabilization has been shown to have be neuroprotective in animal models of neonatal excitotoxicity (Patkai et al. 2001) and hypoxia-ischemia (Jin et al. 2007), as well as in adult TBI (Hendrix et al. 2013) and stroke (Strbian et al. 2006). However, little is known regarding the involvement of mast cells in the injury process triggered by TBI in the developing brain.

In the current article, we studied the role of mast cells in pediatric TBI using animal and cellular models. In rodents, during the first 30 days of life, injury to the brain is maximal when TBI occurs on postnatal day (P) 7 (Bittigau et al. 1999). The first three postnatal weeks also coincide with the infantile period in humans, characterized by several crucial developmental processes (e.g. maximal brain growth, synaptogenesis and myelination), in addition to a heightened sensitivity to excitotoxicity (Bittigau et al. 1999). We first examined whether neural cells in the brain of P7 mice could respond to histamine, the primary molecule released by mast cell degranulation, to determine whether this could be a valid injury mechanism. Next, we examined the effects of cromoglycate, an inhibitor of mast cell degranulation, administered at different time-points relative to TBI, on tissue loss, neuroinflammation and the loss of myelin. Finally, we confirmed our observations in genetically modified mice deficient in cKit, also known as mast cell growth factor receptor or CD117, a receptor tyrosine kinase essential for the differentiation of mast cells.

Materials and Methods

Experimental protocols were approved by the Bichat and Robert Debré Hospital ethics committee (N°2011-14/676-0050). Protocols were as previously described (Chhor et al. 2013; Favrais et al. 2011; Husson et al. 2005; Patkai et al. 2001) and adhered to European Union Guidelines for the Care and Use of Animals. *In vivo* procedures were typically carried out between 10am and 1pm (light phase 7am-7pm daily); all animals were monitored daily during experimentation. Each experimental unit corresponds to a single animal, with groups spread between and across litters. Animals were housed (Plexiglas cages 30x18x15cm) together with littermates and their dam for the entire experiment, with wood-chip bedding and shredded paper for nesting (Pharmaserv, France). Animals had access to standard chow and water *ad libitum*.

Primary microglial, astrocytic and neuronal cultures

Microglia: Primary mixed glial cultures were prepared from the cortices of wild-type OF1 mice (Charles River, L'Arbresle, France) on post-natal days (P) 0-1 as previously described (Chhor et al. 2013; Kaindl et al. 2007). An entire litter was used for a culture (for all cell types), and an approximately equal number of males and females were identified. In brief, the cortices were dissected after removal of the meninges, and mechanically dissociated before resuspension and plating in low-glucose DMEM (31885, Gibco, Cergy Pontoise, France) supplemented with 10% FBS (Gibco) and 0.01% penicillin-streptomycin (Gibco). Microglia were isolated from the primary mixed glial cultures by shaking on day *in vitro* (DIV) 14 and resuspended at a concentration of $4x10^5$ cells/ml in 6-well culture plates. Culture purity was verified by immunolabeling (n=5 wells) using cell-type-specific antibodies to

microglia, astrocytes and neurons as previously described (Chhor et al. 2013) and revealed >99% purity for microglia.

Astrocytes: On DIV14, after shaking off microglia from the primary mixed glial cultures above, the microglial cell medium was removed, the plates were treated for 5 minutes with 0.25% trypsin (Gibco, France), and the remaining cells (astrocytes) resuspended in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum (Gibco, France) with 0.01% of penicillin-streptomycin, at a concentration of $6x10^5$ cells/ml and plated in 6-well culture plates.

Neurons: Primary neuronal cultures were derived from the cerebral cortex of embryonic day (E) 14.5 C57Bl/6 mice as previously described (Chhor et al. 2013). In brief, after dissection of the cortices and removal of the meninges, the cortices were minced, chemically dissociated with 0.0125% trypsin (Gibco) and cells seeded in 96-well culture plates pre-coated with poly-DL-ornithine (Sigma) and laminin 2.5 µg.ml-1 (Sigma) at a density of 5×10^4 cells and a final volume of 100µl per well. Cells were cultured in Neurobasal Medium (Invitrogen, Illkirch, France) supplemented with 2% B27 (Invitrogen), 300µM glutamine (Sigma) and 1% Streptomycin/amphotericin B 100X (Invitrogen). Half the culture medium was replaced by fresh medium three times a week and 5µM arabinocytidine hydrochloride (Sigma) added on DIV3 to kill dividing progenitors.

Primary cultured microglia, astrocytes and neurons were harvested and RNA extracted for gene expression analysis, as described below.

RNA extraction and quantification of gene expression by real-time qPCR

Preparation of microglia qRT-PCR, primer design, and PCR protocol were similar to that previously described (Chhor et al. 2013; Husson et al. 2005; Schang et al.

2014). Primer sequences are given in Table I. *Gapdh* for microglia and neurons and *Rpl13* for astrocytes were used to standardize gene expression levels in quantitative experiments based on prior reference gene suitability testing. The relative quantities are expressed as the ratio between the gene of interest and the reference gene. For characterization of CD11b-positive cells *ex vivo* (see below), genes were grouped into cytotoxic (CytoT), reparatory/regenerative R-Regen) and immunomodulatory (ImmunoM) types, based on standard classification the literature (Colton and Wilcock 2010; Ransohoff and Perry 2009) and previous work from our group (Chhor et al. 2013). Analyses were performed with Bio-Rad CFX manager 2.1 software (RRID:SCR_003375).

MTT mitochondrial activity (cell viability) assays

Primary cultured neurons (DIV11) prepared as above were subjected to a 1-hour treatment with 50µM NMDA followed by a 24-hour treatment with different concentrations of histamine (1, 10, 100µM; Sigma). They were then subjected to the MTT assay, where the bioreduction of MTT, a yellow tetrazolium salt, to formazan by mitochondrial enzymes reflects the proportion of living cells. In brief, MTT solution (Sigma) was added to a final concentration of 0.85mg/ml to each well of a 96-well plate for 1 hour. Medium was then replaced by DMSO (50µl/well). The absorbance of formazan was measured at 560nm and 750nm using the Glomax-Multi detection system (Promega, Southampton, UK). Data were normalized to the vehicle group.

Traumatic brain injury model and experimental procedure

In a TBI model described previously (Kaindl et al. 2007), 7-day-old (P7; weight 4-5g) wild-type OF1 mice of both sexes (Charles River, L'Arbresle, France) were randomly (alternating animals) allocated to TBI, sham control or TBI+ treatment (PBS or drug)

groups. Injury profiles were not different between males and females and data was pooled. In a separate experimental work-space within the animal facility, mice were anesthetized with isoflurane (8% induction) and subjected to a closed head weight–drop head trauma, in a process that took no longer than 3 minutes in total. In brief, the head was fixed to a stereotaxic frame, the skull surface exposed with a skin incision and the contusion device oriented perpendicular to the parietal bone with the center of the foot-plate (2mm diameter) positioned 2 mm anterior and 1 mm lateral to lambda on the parietal bone. The foot-plate was first allowed to touch the skull and was then further depressed by 0.5 mm. The contusion device consisted of a hollow stainless-steel cylinder 20 cm in length, perforated at 1 cm intervals to prevent air compression, and guiding a 10 g weight falling from a height of 10 cm onto the foot-plate. The contusion was delivered unilaterally to the left side of the skull by the same operator for all animals in a given experiment, and cortical contusions were of comparable severity in all animals. Body temperature was kept constant via the use of a heating pad maintained at 37 °C until pups were returned to their dams, approximately 15 minutes post-TBI. A single intraperitoneal injection of cromoglycate (50mg/kg in PBS; Sigma) or PBS was administered 1 hour before or 1 hour after TBI (Fig. 1). All tissue processing and analyses were carried out by investigators blind to the treatment group.

cKit mice

Double-heterozygous cKit$^{Wv/W}$ mice on a C57Bl/6 background (Jackson Laboratory; RRID: IMSR_JAX:000049) as used previously in our lab (Patkai et al. 2001), were subjected to TBI experiments following the protocol described above. In this colony, the homozygous condition is lethal, heterozygotes for both alleles are white in color and lack mast cells, heterozygotes for either allele alone have various degrees of

white spotting and functional mast cells, and wild-type mice are black (Meininger et al. 1992). In order to limit variability between mutant animals and controls, we chose single-heterozygous mice ($cKit^{Wv/+}$ or $cKit^{W/+}$) as controls for mast-cell deficient double-heterozygotes once initial comparisons between $cKit^{Wv/+}$ and $cKit^{W/+}$ mice and between single-heterozygous mice and wild-type mice revealed no significant differences among experimental parameters. Phenotype-genotype correlations were confirmed before experimentation by PCR and restriction enzyme digestion (Nsi1), and revealed a 100% match.

Tissue preparation and histology

Assessment of mast cell numbers: Four hours after TBI, mice were transcardially perfused with 4% paraformaldehyde–0.12M PBS, the scalp was removed and the head with the skull post-fixed in 4% formol overnight (to preserve the meninges) before immersion in a 5% nitric acid bath for 1 hour to decalcify the skull. Brains were then post-fixed in 4% formol for an additional 4 days before embedding in paraffin, 16µm coronal sections cut and collected on Superfrost Plus slides, and stained with 1% Toluidine Blue (Fisher Scientific, Leicestershire, UK), which labels mast cell granules rich in heparin and histamine, as previously described (Patkai et al. 2001). The number of mast cells in the brain and meninges was calculated from counts under a 10X objective on a Leica DM6000 B microscope (Leica Microsystems Ltd.; RRID:SCR_000011) in two entire sections from the area of maximum lesioning, at approximately bregma -1.50mm and -3.50mm. Data shown are average counts per section (both hemispheres) for each treatment group.

Nissl staining and immunohistochemistry: One or five days after TBI, animals were sacrificed by decapitation, brains were immersion fixed (4%formol for 5 days), embedded in paraffin and 16µm coronal sections cut from the frontal pole to the

occipital lobes. One series of sections was stained with cresyl violet for the determination of ventricular area as described previously (Kaindl et al. 2007). In short, the border of each lateral ventricle from three sections at 192µm intervals spanning the hippocampus and mid-striatum was outlined, the cross-sectional ventricular areas determined using ImageJ software (version 1.43; NIH, Bethesda, MD, USA; RRID:SCR_003070), and the ratio between ventricular areas in the traumatized (ipsilateral; left) and non-traumatized hemispheres (contralateral; right) determined as previously described (Kaindl et al. 2012; Turner et al. 2003). No differences were noted in ventricular areas between the contralateral hemispheres of sham and TBI mice. Immunohistochemistry (IHC) was performed as previously described (Fleiss et al. 2012), using the following antibodies (see Table II): rabbit monoclonal anti-Iba1 (1:1000, Wako Chemicals USA), rabbit monoclonal anti-CCasp3 (1:200, Cell Signalling), mouse monoclonal anti-MBP (1:500, Millipore), mouse monoclonal anti-MAP2 (1:2000, Sigma). After overnight incubation with primary antibodies and washing, sections were incubated with appropriate fluorescent secondary antibodies (Vectorlabs, California, USA) at 1:200. The number of immunolabeled cells or area of immunolabeling was assessed as previously described (Fleiss et al. 2012). Briefly, for Iba1 and CCasp3 immunolabeling, two images were captured at the level of maximum lesion (approximately -1.50mm) with a Leica DM6000 B microscope (Leica Microsystems Ltd.; RRID: SCR_000011) using a 10X objective. Counts were made of the number of labeled cells in the parietal cortex and striatum of each hemisphere using ImageJ software, and cell numbers within a given region expressed as cells/mm^2. For MAP2 and MBP, labeling was measured in 4-6 levels per brain (one 16µm-thick section every 576 µm) as previously described (Fleiss et al. 2012). Volumes of MAP2 and MBP-positive brain

tissue were calculated from area measurements according to Cavalieri's principle using the following formula: V = SA × P × T, where V is the total volume, SA is the sum of the areas measured, P is the inverse of the sampling fraction and T is section thickness. The difference between the two hemispheres was expressed as the ratio between values for the ipsilateral and contralateral hemispheres.

Isolation of CD11b-positive cells using magnetic bead-coupled antibodies (MACS)

At 24 hours and 5-days following TBI, CD11b-positive cells were extracted from the whole cortex of unfixed brains using a magnetic bead-coupled antibody system (MACS: Miltenyi Biotec, Bergisch Gladbach, Germany; RRID: AB_244268). In brief, the olfactory bulbs and cerebellum were removed, and for each data point, 3-4 hemispheres were pooled (for sufficient RNA yields) and mechanically and enzymatically digested using the Neural Tissue Dissociation Kit (Miltenyi Biotec). Homogenized and digested tissue was incubated with PE-conjugated anti-CD11b antibodies, and CD11b+ cells were separated in a magnetic field before being counted and frozen at -80°C. The purity of the separation was assessed using qRT-PCR for GFAP (astrocytes), MBP (oligodendrocytes), NeuN (neurons) and CD11b (microglia) and showed levels of contamination of less than 5%.

Statistics

Data are from three or more independent experiments and are presented as means ± SEM. No animals were excluded from the analysis. The appropriate statistical tests were chosen based on data normality (Kolmogorov-Smirnov test). A one-way Anova was used to compare qRT-PCR results. Mann-Whitney U tests or Kruskall-Wallis tests followed by Dunnett's post hoc test were used for all other experiments.

The statistical test (performed with GraphPad 5.0 software [San Diego, CA, USA], RRID: SCR_002798) performed on each data set is indicated in the figure legend or within the text and statistically significant results (threshold set at $p \leq 0.05$) indicated by asterisks.

Results

Histamine receptors are present on neural cells, and histamine aggravates excitotoxic neuronal death

As a first step in determining any role of mast cell degranulation in TBI, we asked whether various neural cell types could respond to histamine, one of the principal immunomodulatory substances released by mast cells. Quantitative RT-PCR for histamine receptors 1-4 revealed the expression of several receptors, with the particular abundance of the histamine H2 receptor (H2R), in primary cultures of neurons, microglia and astrocytes (Fig. 2A-C) (n=3). We next asked whether histamine could affect neuronal death due to excitotoxicity, one of the major injury mechanisms involved in TBI, by treating primary neuronal cultures (DIV11) with the glutamate agonist N-methyl D-aspartate (NMDA). MTT assays for cell viability revealed that NMDA-mediated neuronal death was aggravated in a dose-dependent manner by histamine (Fig. 2D) (One way ANOVA; $F(3;76) = 25.27$, n=16-24 wells/group (n=5-6 independent cultures); NMDA vs. NMDA + histamine 1µl: non-significant; NMDA vs. NMDA + histamine 10µl, $p < 0.001$; NMDA vs. histamine 100µl, $p < 0.001$; in Kruskall-Wallis post-test). Histamine added to healthy neuronal cultures did not significantly affect their viability (data not shown). Together, these data suggest that histamine released by mast cell degranulation could indeed play a role in the pathogenesis of TBI, either by directly modulating excitotoxic neuronal death or by acting through glial cells such as microglia or astrocytes, activated as part of the injury response.

Cromoglycate administration before TBI prevents mast cell degranulation and modifies neuroinflammatory processes

Next we assessed whether an inhibitor of mast cell degranulation, cromoglycate, could enter the brain and prevent the deleterious consequences of TBI. In OF1 pups at P7, TBI induced a significant increase in the number of Toluidine-Blue-stained mast cells in the brain (cortex, striatum, thalamus) and meninges 4h after injury when compared to sham-operated pups, suggesting an influx of circulating mast cells (Fig. 3A,B) (Sham vs. TBI, 2.1±0.4 vs. 8.8±1.9 in the brain parenchyma (U=19.5, n=7, p=0.0009) and 5.4±0.8 vs. 10.3±1.6 in the meninges (U=35.5, n=7, p=0.01), respectively; Mann-Whitney U test). Cromoglycate 50mg/kg administered intraperitoneally 1h before TBI further increased the number of Toluidine-Blue-positive mast cells in the meninges (Fig. 3C,D) (U=27, n=12; p=0.004; Mann-Whitney U test), with a similar but non-significant increase in the brain.

Since one of the mechanisms triggered by TBI is microglial activation and neuroinflammation, we then used quantitative RT-PCR at 24h to evaluate the expression of a previously validated panel of microglial phenotype markers (Chhor et al. 2013) (broadly divided for ease of analysis into CytoT (cytotoxic), R-Regen (reparatory/regenerative) or ImmunoM (immunomodulatory) types), as well as cytokines/chemokines on MACS-sorted CD11b-positive cells (verified to be >95% pure for microglia) to assess the effect of cromoglycate on microglial activation (Fig. 4). Cromoglycate significantly reversed the TBI-induced increase in two markers of cytotoxic phenotype activation in microglia, iNOS ($F(9;3.50)$, n=5-6, p=0.007) and Cox-2 ($F(9;2.47)$, n=5-6, p=0.04), and the TBI-induced decrease of two others, CD32 ($F(9;5.90)$, n=5-6, p=0.0002) and CD86 ($F(9;2.36)$, n=5-6, p=0.04) (Fig. 4A, top panel) (n=5-6; one-way Anova with Dunnett's post hoc test). Whereas no reparatory/regenerative phenotype markers studied were affected (Fig. 4A, middle panel), cromoglycate further reduced two immunomodulatory phenotype markers

whose expression was reduced by TBI – SOCS3 (F(9;2.67), n=5-6, p=0.03) and IL-4Rα (F(9;2.23), n=5-6, p=0.05) (Fig. 4A; bottom panel) (n=5-6; one-way Anova with Dunnett's post hoc test). Among the cytokines/chemokines assayed, cromoglycate significantly reduced levels of the pro-inflammatory cytokine TNFα (F(9;2.20), n=5-6, p=0.05), (reversing its TBI-induced increase), and reduced levels of the chemokine CCL3 (or MIP1α; F(9;3.22), n=5-6, p=0.01), in addition to further reducing levels of the anti-inflammatory cytokine IL-10 (Fig. 4B) (F(9; 2.84), n=5-6, p=0.02; All analysis via one-way Anova with Dunnett's post hoc test). To summarize, cromoglycate administration before TBI did indeed inhibit TBI-induced mast cell degranulation as well as altering the inflammatory status of activated microglia.

Cromoglycate administration after TBI does not prevent tissue loss or neuropathological changes at the cellular level.

Since any neuroprotective molecule destined for therapeutic application needs to be effective when administered after injury, we assessed the effects of cromoglycate given 1h after TBI. As an index of tissue loss following TBI, ventricular dilatation was assessed + 1 day and + 5 days after TBI (i.e. on P8 and P12) by calculating the size of the ventricle in the lesioned ipsilateral hemisphere as a percentage of the size of the ventricle of the contralateral hemisphere in cresyl-violet-stained sections (Fig. 5A,B). TBI induced an increase in ventricular volume on the ipsilateral side compared to the contralateral side at + 5 days post-TBI. However, cromoglycate treatment did not significantly alter ventricular volumes at + 1 day (U=3, n=4-6; p=0.07) or 5 days (Fig. 5C,D) (n=9-10; p=0.56; Mann-Whitney U test). TBI also induced significant cellular labeling for cleaved caspase-3 (CCasp3) on + 1 day in the cortex and striatum underlying the contusion site (Fig. 5E,F) (U=0, n=4-6; p=0.002 for both regions; Mann-Whitney U test), as well as other brain regions

(thalamic nuclei, hippocampal dentate gyrus and subiculum; not shown). In agreement with ventricular volumes, however, cromoglycate did not significantly reduce CCasp3 density on the ipsilateral side (Fig. 5E,F) (n=4-6; p=0.17, cortex; p=1.0, striatum; Mann-Whitney U test; differences were also non-significant for the contralateral side). Sham groups not subjected to TBI and treated with vehicle and/or cromoglycate displayed no change in ventricular size and had very low numbers of CCasp3-positive cells (data not shown).

Next, we asked whether the changes in neuroinflammation-related molecules observed following cromoglycate treatment in MACS-sorted microglia were reflected in the number of microglia, or subsequent neuronal death and myelination deficits. The density of Iba1-positive microglia observed by immunohistochemistry on + 1 day following TBI (i.e. P8) was slightly increased in the cortex and striatum of the ipsilateral side when compared to the contralateral side in PBS-treated TBI animals (U=1, n=5; p=0.02, cortex; p=0.05, striatum; Mann-Whitney U test). However, cromoglycate treatment did not significantly modify microglial numbers (Fig. 6A,B) (n=4; p=0.33, cortex; p=0.41, striatum; Mann-Whitney U test). Furthermore, on + 5 days after TBI (i.e. P12), there was no observable effect of cromoglycate on the area of immunolabeling for the neuronal marker MAP2 (p=0.72) or the myelin marker MBP (myelin basic protein; p=0.08) on the ipsilateral side compared to the contralateral side (Fig. 6C,D) (n=9-10; Mann-Whitney U test). The density of Iba1-positive microglia and GFAP-positive astrocytes was also quantified on + 5 days, and revealed no change with cromoglycate treatment (data not shown). Altogether these data indicate that inhibiting mast cell degranulation 1h after TBI did not protect against inflammatory microglial activation, neuronal death or myelination damage.

Cromoglycate administration before TBI does not prevent ventricular dilatation or apoptosis

We noted above a discrepancy between the effects of cromoglycate on the activation of microglia (observed in *ex vivo* MACS-sorted CD11b-positive cells) and the lack of a significant effect on cellular apoptosis and tissue loss *in vivo*. As such, we asked whether mast cell degranulation, which is an extremely rapid event, might exert greater effects at a very early post-injury stage, necessitating cromoglycate treatment before TBI for any improvement in neuropathology to be observable. We therefore repeated the experiment above but with cromoglycate administration 1h before TBI, and measured changes in ventricular volume on + 1 day and + 5 days and CCasp3-positive cells on + 1 day. While these results were still not significant, cromoglycate administered before TBI, did appear to increase tissue loss as measured by ventricular dilatation on + 5 days, but not at + 1 day (Fig. 7A,B) (n=7-10, p=0.27 for + 1 day; n=5-6, p=0.66 for + 5 days; Mann-Whitney U test). The density of apoptotic CCasp3-positive cells in the cortex or striatum of pups on + 1 day was also unchanged on the ipsilateral side (Fig. 7C,D) (n=7-10; p=0.47, cortex; p=0.70, striatum; Mann-Whitney U test; differences were also non-significant for the contralateral side). Altogether these data indicate that blocking mast cell degranulation before TBI was insufficient to affect TBI outcome.

Mast-cell-deficient mice do not display altered neuropathological changes following TBI

Finally, we aimed to verify that it was not a dose- or treatment-regimen-specific effect that led us to see no effect of targeting mast cells using cromoglycate in our pediatric TBI model. As such, we used double-heterozygous mice (cKit$^{Wv/W}$) lacking

functional cKit leading to mast cell depletion, with single-heterozygous littermates as controls. $cKit^{Wv/W}$ mice subjected to TBI at P7 and sacrificed 1 day later showed no difference in the TBI-induced increase in ventricular volume when compared to single-heterozygous mice ($cKit^{Wv/+}$ or $cKit^{W/+}$) (Fig. 8A) (n=9-13; p=0.23; Mann-Whitney U test), but did show a significant increase in ventricular volume when sacrificed at + 5 days (Fig. 8B) (U=11, n=7-10; p=0.02; Mann-Whitney U test). The density of CCasp3-positive cells was not significantly different between the cortex and striatum of $cKit^{Wv/W}$ mice and single-heterozygous controls on + 1 day (Fig. 8C,D) (n=9-13; p=0.14, cortex; p=0.36, striatum; Mann-Whitney U test).

In addition, $cKit^{Wv/W}$ mice were also not significantly different from their single-heterozygous littermates in terms of the density of Iba1-positive microglia in the cortex or striatum at + 1 day (Fig. 9A,B) (n=9-13; p=0.42 for the cortex; p=0.76 for the striatum; Mann-Whitney U test). Similarly, on + 5 days, both groups of mice had a similar area measurements of MAP2 immunolabeling as a proxy of neuronal number (Fig. 9C) (n=7-10; p=0.47; Mann-Whitney U test) and MBP immunolabeling as a measure of myelination (Fig. 9D) (n=7-10; p=0.89; Mann-Whitney U test). To summarize, the absence of mast cells, while increasing ventricular dilatation at the late but not early time point assessed, did not significantly influence cell death, microglial number or surrogates of myelination or healthy mature neurons following TBI.

Discussion

Previous observations indicate that mast cells infiltrate the brain and degranulate following adult TBI (Levy et al. 2015; Lozada et al. 2005), which triggers neuronal death, reactive gliosis, blood brain barrier opening, and myelination deficits in the brain through inflammatory and excitotoxic pathways (Stokely and Orr 2008). As such, we examined the possibility that mast-cell-released molecules could influence the injury mechanisms involved in a model of pediatric TBI. Our results show that several neural cell types express receptors for histamine, one of the main molecules released by mast cells and a mediator of inflammation, and that histamine exacerbates excitotoxic cell death in primary neuronal cultures. Furthermore, cromoglycate, an inhibitor of mast cell degranulation, when administered peripherally before TBI increased the numbers of toluidine blue positive mast cells (as previously reported in rats (Dong et al. 2016; Strbian et al. 2007)). This suggests that cromoglycate effectively inhibited degranulation of the mast cells that infiltrated the brain following TBI. However, we did not specifically count the degranulated cells due to difficulties in finding these ‘ghost’ cells as after they release their histamine granulates these dissipate. As such, we can only make assumptions that increases in number reflect decreased degranulation as previously reported (Levy et al. 2015). Cromoglycate has limited but significant effects on markers and cytokines typical of activation states of microglia, the principal mediators of inflammation in the brain. However, regardless of whether cromglycate was administered 1h before or 1h after TBI, it did not affect TBI-induced ventricular dilatation, apoptosis, increased microglial numbers or measures of neuronal density or myelination. Finally, the lack of an effect of stabilizing mast cells using a pharmacological approach on outcome in this model of pediatric TBI can be largely reproduced in a transgenic mouse line in

which mice lack functional cKit and as such have no mast cells. It thus appears that the influx of mast cells following TBI in the pediatric brain is either not sufficient to influence the majority of the neuropathological mechanisms triggered, or has effects limited to parameters that were not measured in our experiments.

This is the first study to examine the role of mast cells in a pediatric TBI model. However, we have previously identified a role for these cells in a model of excitotoxicity in the immature brain (Mesples et al. 2005; Patkai et al. 2001), and cromoglycate has been shown to significantly reduce lesion size in a neonatal model of hypoxia-ischemia (Jin et al. 2007). We chose a closed-contusion weight-drop model of TBI in P7 mice for this study, as the injury mechanisms triggered in this model are similar to those seen in pediatric TBI (Xiong et al. 2013). Our observations on the distribution and timing of cell death are generally in agreement with previous studies using a similar closed-contusion model in neonatal rodents (Bittigau et al. 1999; Kaindl et al. 2007). In an adult TBI model using the same mast-cell-deficient (cKit knockout) mice used in this study (Hendrix et al. 2013), mast cells were found to be protective, and associated with reduced lesion size, astrogliosis and T cell infiltration. In our pediatric model, however, apart from an indication that TBI-induced ventricular dilatation could be aggravated in the absence of mast cells, the cellular and myelination markers measured were not affected in cKit mice. There are several physiological reasons why the effects of mast cells in TBI might differ between the adult and neonatal brain, including i) the substantially greater number of mast cells in the brain of neonatal rodents and humans compared with adults (Dropp 1979; Khalil et al. 2007), ii) the differential effects of mast cell granular contents on the vasculature (Ribatti 2015; Stolp et al. 2005), and iii) the developmental regulation of

mast cell phenotype, including the types of chymases produced (Dimitriadou et al. 1996). Indeed, mast cell granules contain a variety of molecules in addition to histamine – heparin, various cytokines as well as several chymases etc. – capable of mediating either injury or neuroprotection, and the exact effects of their degranulation could depend on both age and the injury context (Hendrix et al. 2013; Piliponsky et al. 2012). Further in-depth mechanistic studies are required to elucidate the differences in the underlying neuropathology between hypoxic/ischemic and excitotoxic injury versus TBI in neonates, and the role of mast cells in these differences.

The specificity of cromoglycate as a stabilizer of mast cells in mice is contested but it is acknowledged that even in rats where this drug is considered mast cell specific that subpopulations of mast cells respond very differently to cromoglycate exposure (Chen et al. 2001). In addition, as mentioned above that there are striking differences between neonatal and adult mast cells that may influence specificity and effect size (Dimitriadou et al. 1996; Khalil et al. 2007; Monk et al. 2007), and these variables have not been addressed in the literature regarding the differences between mice and rat responses to cromoglycate. Furthermore, supporting the efficacy of cromoglycate in mice is that it has been demonstrated to reduce IgM mediated autoimmunity in a mouse model, an effect not observed in cKit mast cell KO mice (Chen et al. 2001); in a mouse model of peripheral nerve injury cromoglycate prevented mast cell recruitment into the injury (Monk et al. 2007); and we noted that treatment increased the number of mast cells, suggesting they were prevented from degranulation due to TBI. Together with the strong safety profile of cromoglycate in the pediatric population this previous work using cromoglycate in mice justifies the use of this compound in this study.

We observed mast cell recruitment primarily in the meninges as previously reported in an adult model of TBI (Shimada et al. 2012) although we did not count degranulated ‘ghost’ mast cell numbers to directly replicate the observation that in addition to recruitment there is degranulation of mast cells, such as after adult TBI (Lozada et al. 2005) or neonatal hypoxia-ischemia (Biran et al. 2008; Jin et al. 2009). We deduce from the further increase in mast cell numbers from TBI only levels caused by cromoglycate that degranulation of these recruited mast cells was prevented. However, unlike previous reports, we did not observe an increase in microglial number following the inhibition of mast cell degranulation (Hendrix et al. 2013). Cromoglycate was able to generally reduce the pro-inflammatory associated markers in TBI-activated microglia (i.e., reducing iNOS, COX2 and TNFa). However, the response of microglia was small overall in response to TBI as compared to insults such as lipopolysaccharide exposure (Smith et al. 2014). In contrast to our observations an anti-inflammatory effect of histamine on activated microglia has previously been reported (Ferreira et al. 2012).

In summary, mast cells infiltrate the brain and degranulate following TBI at P7. However, despite some modulatory effects of mast cell degranulation on the inflammatory phenotype of microglia, pharmacologically suppressing mast cell activity or genetically eliminating mast cells had little effect on tissue loss or associated cellular and neuroanatomical changes in our model. This suggests that compared to the adult brain, the effects of mast cells in TBI during development may be limited and not a viable target as a neurotherapeutic.

Acknowledgements

For work relating to the setup of the TBI model we thank Dr Angela Kaindl and Professor Chris Ikonomidou.

Conflict of Interest Statement

All authors declare that there are no conflicts of interest

Role of Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: RM, VC, VD, LT, PG, BF. Technical and material support and acquisition of data: RM, VC, DB, EB, SDL, TLC, SL, LS, JP. Analysis and interpretation of data: RM, VC, TLC, SL, LS, JP, VD, LT, PG, BF. Drafting of the manuscript: RM, VC, SR, VD, LT, PG, BF. Critical revision of the manuscript for important intellectual content: RM, VC, SR, VD, LT, PG, BF. Statistical analysis: RM, VC, PG, BF. Obtained funding: VD, PG, BF. Study supervision: VD, LT, PG, BF.

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Table I: Primer sequences and NCBI references

Gene	Sense	Anti-sense	NCBI Reference
Gapdh	GGC CTT CCG TGT TCC TAC	TGT CAT CAT ATC TGG CAG GTT	NM_008084.2
Rpl13	ACA GCC ACT CTG GAG GAG AA	GAG TCC GTT GGT CTT GAG GA	NM_016738.5
iNos	CCC TTC AAT GGT TGG TAC ATG G	ACA TTG ATC TCC GTG ACA GCC	NM_010927.3
CD32	CTG GAA GAA GCT GCC AAA AC	CCA ATG CCA AGG GAG ACT AA	NM_010187.2
CD86	GAG CGG GAT AGT AAC GCT GA	GGC TCT CAC TGC CTT CAC TC	NM_019388.3
Ptgs2	TCA TTC ACC AGA CAG ATT GCT	AAG CGT TTG CGG TAC TCA TT	NM_011198.3
CD206	CTT CGG GCC TTT GGA ATA AT	TAG AAG AGC CCT TGG GTT GA	NM_008625.2
Arg1	GTG AAG AAC CCA CGG TCT GT	GCC AGA GAT GCT TCC AAC TG	NM_007482.3
Lgals3	GAT CAC AAT CAT GGG CAC AG	ATT GAA GCG GGG GTT AAA GT	NM_010705.3
Igf1	TGG ATG CTC TTC AGT TCG TG	GCA ACA CTC ATC CAC AAT GC	NM_010512.4
Sphk1	TCC AGA AAC CCC TGT GTA GC	CAG CAG TGT GCA GTT GAT GA	NM_001172475.1
Il1rn	TTG TGC CAA GTC TGG AGA TG	TTC TCA GAG CGG ATG AAG GT	NM_031167.5
Il4ra	GGA TAA GCA GAC CCG AAG C	ACT CTG GAG AGA CTT GGT TGG	NM_001008700.3
Socs3	CGT TGA CAG TCT TCC GAC AA	TAT TCT GGG GGC GAG AAG AT	NM_007707.3
IL1b	GGG CCT CAA AGG AAA GAA TC	TCT TCT TTG GGT ATT GCT TGG	NM_008361.3
IL-6	CAA AGC CAG AGT CCT TCA GA	GCC ACT CCT TCT GTG ACT CC	NM_031168.1
IL10	CTC CCC TGT GAA AAT AAG AGC	GCC TTG TAG ACA CCT TGG TC	NM_010548.2
IL-12a	TCA CAA CCA TCA GCA GAT CA	TGC AGA GCT TCA TTT TCA CTC	NM_001159424.1
IL-12b	ATC CAG CGC AAG AAA GAA AA	AAT AGC GAT CCT GAG CTT GC	NM_008352.2
IL-18	TTC GTT GAC AAA AGA CAG CC	TAT CAG TCT GGT CTG GGG TTC	NM_008360.1
Tnfa	GCC TCT TCT CAT TCC TGC TT	AGG GTC TGG GCC ATA GAA CT	NM_013693.3
Cxcl1	GCA CCC AAA CCG AAG TCA TA	AGG TGC CAT CAG AGC AGT CT	NM_008176.3
Cxcl10	GGG TAA AGG GAG GTG GAG AG	GCT TAT TGA AAG CGG TGA GC	NM_021274.2
Ccl2	CAT CCA CGT GTT GGC TCA	TCA TTG GGA TCA TCT TGC TG	NM_011333.3
Ccl3	TTT TGA AAC CAG CAG CCT TT	CTG CCT CCA AGA CTC TCA GG	NM_011337.2
Hrh1	GGG CTC AAA GGC CAA TGA C	ACT GTC GAT CCA CCA AGG TC	NM_008285.3
Hrh2	CTG GCT GTC AGC TTG AAT CG	CCA AAG CTC CAC TTG AAG GA	NM_001010973.2
Hrh3	CCA TCT CCG ACT TCC TCG T	AGT CTA CCA CCA GCC ACA GC	NM_133849.3
Hrh4	GCT ACG ATC GAT ACC AGT CA	AAG AAA GCC AGT ATC CAA ACA G	NM_153087.2
Mbp	CCG GAC CCA AGA TGA AAA C	CTT GGG ATG GAG GTG GTG T	NM_010777.3
Gfap	CTC CTG GTA ACT GGC CGA CT	AAG CCA AGC ACG AAG CTA AC	NM_010277.3
NeuN	CGA TGC TGT AGG TTG CTG TG	CAG ATA TGC TCA GCC AGC AG	NM_001039168.1
CD11b	CTG GTG CTC TTG GCT CTC AT	GGC AGC TTC ATT CAT CAT GT	NM_001082960.1

Table II. Antibodies used

Name of antibody	Immunogen	Manufacturer, catalog number, RRID, monoclonal/polyclonal antibody, species	Concentration
Anti Iba-1 (Ionized calcium-binding adaptor molecule 1)	Iba1 carboxy-terminal sequence	Wako, 019-19741, AB_839504, polyclonal antibody, Rabbit	1:1000
Anti Cleaved Caspase-3	Amino-terminal residues adjacent to (Asp175) in human caspase-3	Cell signaling, 9661, AB_331440, monoclonal antibody, Rabbit	1:200
Anti-MBP (Myelin Basic Protein)	Amino acids 129-138 of Myelin Basic Protein	Millipore, MAB382, AB_94971, monoclonal antibody, Mouse	1:500
Anti-MAP2 (Microtubule-associated protein-2)	Rat brain microtubule associated proteins (MAPs)	Sigma-Aldrich, M4403, AB_477193, monoclonal antibody, Mouse	1:2000

Figure Legends

Figure 1. Schematic representation of experimental protocols. Cromoglycate was administered either before or after TBI. cKit mice underwent TBI but not cromoglycate treatment. CCasp3: cleaved caspase 3; Iba1: ionized calcium-binding adaptor molecule 1 (microglial marker); MAP2: microtubule-associated protein 2 (neuronal marker); MBP: myelin basic protein (marker of myelination/myelinating oligodendrocytes); TBI: traumatic brain injury.

Figure 2. Histamine receptor expression in neural cells and histamine-mediated potentiation of excitotoxic neuronal death. Expression of histamine receptor subunits in primary cultured microglia (A), astrocytes (B) and neurons (C) as measured by qRT-PCR. D) Effects of histamine on NMDA-mediated neuronal death *in vitro*. Data expressed as mean±SEM (n=16 wells/group in 3 different experiments). ***: p <0.001; one-way ANOVA with Dunnett's post hoc test, compared to NMDA treatment only.

Figure 3. Induction of mast cell recruitment by TBI and prevention of mast cell degranulation by cromoglycate. A) Representative images of Toluidine-Blue-positive mast cells in the periventricular parenchyma at bregma 1.5, scale bar: 100µm. B) Quantification of Toluidine-Blue-positive cells in the meninges and total brain parenchyma. Data expressed as mean±SEM (n=7 animals/group) and groups were compared with a Mann-Whitney U test. *: p<0.05, **: p<0.01. ***: p<0.001.

Figure 4. Effects of pre-TBI cromoglycate treatment on expression of microglial phenotype markers and cytokines/chemokines. Data are shown normalized to expression levels in the sham group (dotted line) and expressed as mean±SEM (n=5-6 mice per group). Genes are grouped based on predicted role in

inflammation: cytotoxic (CytoT), reparatory/regenerative (R-Regen) and immunomodulatory (ImmunoM), with PBS-treated groups in white and cromoglycate-treated (Cromo) groups in shades of grey. Groups were compared using one-way Anova with Dunnett's post test. *: $p<0.05$. **: $p<0.01$. In MACS-sorted CD11b+ microglia at + 1 day post-TBI A) microglial phenotype markers and B) microglial cytokine/chemokine gene expression.

Figure 5. Effects of post-TBI cromoglycate administration on neuropathology. A, B) Representative images of cresyl violet stained sections from animals treated with PBS (A) or cromoglycate (B) 1h after TBI, indicating ventricular cross-sectional area (green: right/contralateral hemisphere; red: left/ipsilateral hemisphere). Ventricular dilatation was used as an index of tissue loss. C, D) Quantification of ventricular area on + 1 day (C) and + 5 days after TBI (D). E, F) Quantification of density of cleaved caspase 3 (CCasp3)-positive cells in PBS- versus cromoglycate-treated animals in the cortex (E) and striatum (F). Data expressed as mean±SEM (n=4-6 for +1 day; n=9-10 for + 5 days); groups were compared using a Mann-Whitney U test. There were no significant differences other than between ipsilateral and contralateral hemispheres (not indicated).

Figure 6. Effects of post-TBI cromoglycate administration on microglial, neuronal and myelin markers. A, B) Quantification of the density of Iba1-positive cells on + 1 day and + 5 days in PBS- and cromoglycate-treated (1h after TBI) mice within the cortex (A) and striatum (B). C) Quantification of neuronal loss on + 5 days in PBS- and cromoglycate-post-treated mice based on the ratio of the area of MAP2 immunoreactivity between the traumatized (ipsilateral; left) and non-traumatized (contralateral; right) hemispheres. D) Quantification of myelin loss on + 5 days, in PBS- and cromoglycate-treated mice based on the ratio of the area of MBP

immunoreactivity between the traumatized (ipsilateral; left) and non-traumatized (contralateral; right) hemispheres. Data expressed as mean±SEM (n=4 per group for + 1 day; n=9-10 per group for + 5 days). Groups were compared using a Mann-Whitney U test. There were no significant differences other than between ipsilateral and contralateral hemispheres (not indicated).

Figure 7. Effects of pre-TBI cromoglycate administration on neuropathology. A, B) Quantification of ventricular area on + 1 day (A) and + 5 days (B) in PBS- and cromoglycate-pretreated (1h before TBI) mice. C, D) Quantification of density of cleaved caspase 3 (CCasp3)-positive cells in PBS- versus cromoglycate-treated animals in the cortex (C) and striatum (D). Data indicate means ± SEM (n=7-10 for + 1 day; n=5-6 for + 5 days). Groups were compared using a Mann-Whitney U test. There were no significant differences other than between ipsilateral and contralateral hemispheres (not indicated).

Figure 8. Effects of genetic deletion of mast cells on TBI-induced neuropathology. A, B) Quantification of ventricular area on + 1 day (A) and + 5 days (B) after TBI. C, D) Quantification of density of cleaved caspase 3 (CCasp3)-positive cells in mast-cell deficient double-heterozygous mice ($cKit^{Wv/W}$) versus single-heterozygous controls ($cKit^{Wv/+}$ or $cKit^{W/+}$) in the cortex (C) and striatum (D). Data indicate means ± SEM (n=9-13 for + 1 day; n=7-10 for + 5 days). Comparisons were carried out using a Mann-Whitney U test. *: p=0.02 There were no other significant differences, except for differences between ipsilateral and contralateral hemispheres (not indicated).

Figure 9. Effects of genetic deletion of mast cells on post-TBI microglial, neuronal and myelin markers. A, B) Quantification of the density of Iba1-positive

cells on + 1 day in mast-cell deficient double-heterozygous mice ($cKit^{Wv/W}$) and single-heterozygous controls ($cKit^{Wv/+}$ or $cKit^{W/+}$) within the cortex (A) and striatum (B). C) Quantification of neuronal loss on + 5 days in mast-cell deficient double-heterozygous mice ($cKit^{Wv/W}$) versus single-heterozygous controls ($cKit^{Wv/+}$ or $cKit^{W/+}$) based on the area of MAP2 immunoreactivity between the traumatized (ipsilateral; left) and non-traumatized (contralateral; right) hemispheres. D) Quantification of myelin loss at + 5 days post-TBI, in mast-cell deficient double-heterozygous mice ($cKit^{Wv/W}$) versus single-heterozygous controls ($cKit^{Wv/+}$ or $cKit^{W/+}$) based on the ratio of the area of MBP immunoreactivity between the traumatized (ipsilateral; left) and non-traumatized (contralateral; right) hemispheres. Data indicate means ± SEM (n=9-13 for + 1 day; n=7-10 for + 5 days). Comparisons were carried out using a Mann-Whitney U test. There were no significant differences other than between ipsilateral and contralateral hemispheres (not indicated).